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14. ABSTRACT

RNA interference (RNAi) is a gene silencing pathway with roles in mRNA stability, translational control, chromatin organization and genome regulation. MicroRNAs (miRNAs) are a set of small RNAs produced by the RNAi machinery that play important functions in tissue organization and maintenance of cell identity. Several miRNAs have been shown to collaborate with oncogenes in the progression of cancer, and in addition, miRNA expression profiling has revealed widespread miRNA misregulation in cancer. To address the role of miRNAs in the onset and maintenance of cancer, we have created embryonic stem (ES) cells and mice in which Dicer, a key enzyme in miRNA biogenesis, can be conditionally inactivated. We have demonstrated loss of Dicer delays onset of teratomas derived from subcutaneously injected ES cells. In addition, we have identified a miRNA family that is regulated by the master tumor-suppressor gene p53. We show that the miR-34 family is a direct target of p53 transcriptional regulation. Our work places miR-34 in the p53 tumor suppressor network, implicated in many cancers, including breast cancer.

15. SUBJECT TERMS
Dicer, RNAi, mammary stem cells, microRNA

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Introduction

The important role of small RNAs in cancer is becoming ever more widely appreciated. Small RNAs, particularly microRNAs (miRNAs) have been identified in networks that both promote and protect against tumor growth (Bagnyukova et al 2006; Kent & Mendell 2006). Using genetic approaches, we have succeeded in gaining further insight into the mechanisms by which RNA interference pathways and oncogenic pathways intercept.

RNA interference (RNAi) is broadly used to describe gene silencing pathways that encode specificity information in small RNAs. The flexibility of the system has led to its adaptation to diverse biological niches. Although its ancestral role remains uncertain, RNAi has evolved to remarkable complexity in both form and function, and provides interesting examples of both convergent and divergent evolutionary routes. We have taken a genetic approach to understanding the actions of small RNA biology in cancer by creating mouse and cell-based models lacking *Dicer*, a ribonuclease essential for small RNA biogenesis in many well-known RNAi pathways. In addition, we have identified a miRNA family that is regulated by the tumor-suppressor gene *p53*.

MiRNAs are small 18-22 nt RNAs derived from foldback hairpin precursors that regulate endogenous target genes at the post-transcriptional level. In animals, miRNAs are often located in polycistronic clusters that are frequently embedded in introns of genes (Murchison & Hannon 2004; Rodriguez et al 2004). MiRNAs are often under strong developmental- and tissue-specific control, are transcribed by either RNA polymerase II or III (Borchert et al 2006; Cai et al 2004; Lee et al 2004). Although many miRNAs in both plants and animals are conserved at the level of both sequence and function, new sequencing technologies have enabled the discovery of increasing numbers of non-conserved miRNAs (Axtell & Bartel 2005; Bartel 2004; Fahlgren et al 2007; Rajagopalan et al 2006). Primary miRNA transcripts fold into imperfect hairpin structures that are consecutively processed into pre-miRNAs and mature miRNAs by the activity of RNase III family members (Murchison & Hannon 2004). In animals, miRNA biogenesis steps are physically separated, as pre-miRNA Drosha products are exported from the nucleus to the cytoplasm by exportin-V, where biogenesis is completed by Dicer (Murchison & Hannon 2004). Mature miRNA duplexes are finally unwound and loaded into Argonaute proteins, where they confer sequence-specificity to RNA induced silencing (RISC) complexes that seek and regulate target genes.

Animal miRNAs have come to heavily rely on a degenerate code for target recognition, which depends predominantly upon sequence complementarity in the six to eight nucleotide 5' "seed" region of the miRNA with the target gene 3'UTR (Bartel 2004). MiRNA regulation in animals can be administered by mRNA cleavage, mRNA sequestration, mRNA degradation or inhibition of translation (Bartel 2004; Valencia-Sanchez et al 2006). Although the mechanism is incompletely understood, it is likely that miRNA regulation is context-dependent, and factors such as miRNA target site sequence context, degree of 3' end complementarity, iteration of target sites, mRNA target structure, miRNA:target molar ratio, titration of secondary factors and mRNA localization may influence degree and mode of regulation (Bartel 2004).

The promiscuous nature of the interaction between animal miRNAs and their targets has applied intense selective pressure on mRNAs to either cultivate or avoid miRNA recognition sequences in their 3'UTRs (Farh et al 2005; Stark et al 2005). Although a single miRNA is likely capable of regulating a vast number of targets, it somewhat paradoxically appears that miRNAs and their targets are frequently expressed in mutually exclusive domains (Farh et al 2005; Stark et al 2005). Thus miRNAs may protect tissues from aberrant expression of undesirable genes, safeguarding tissue identity and integrity, and accelerating the destruction of transitional transcripts (Bartel 2004). Our studies on the role of *Dicer* and small RNAs in cancer biology has directly implicated RNAi in pathways that control tumor growth, most significantly by the identification of a miRNA family that is regulated by the tumor suppressor *p53*.

Body

Delayed teratoma development by *Dicer*$^{-/-}$ ES cells

The establishment of cell lines lacking Dicer offers the opportunity to analyze the role of Dicer and its products in fundamental cellular processes, including predisposition to cancer. We used gene targeting to introduce a Dicer conditional allele into embryonic stem (ES) cells already heterozygous for the Dicer loss-of-function allele to create Dicer conditional ES cells. Cre was introduced transiently to these cells by electroporation to create a subpopulation of Dicer null cells. By monitoring the contribution of the Cre-excised allele to the pool, it was clear that these cells were under negative selection. To isolate pure populations of Dicer null cells, we plated Cre-transfected cells at clonal density and subsequently genotyped individual clones with respect to their Dicer alleles. It was soon apparent that ES cells lacking Dicer failed to proliferate (Murchison et al 2005).

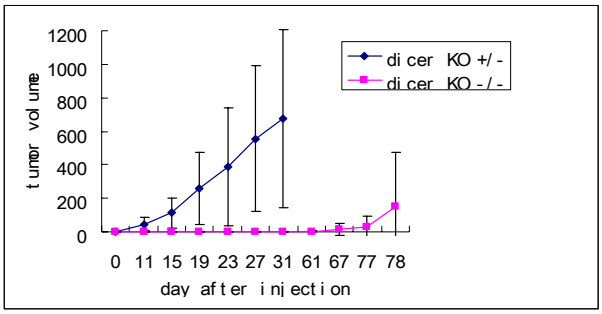

Figure 1: Dicer-/- ES cells are delayed in teratoma formation. ES cells were injected subcutaneously into nude mice. Teratoma growth was monitored at regular intervals by measurement of tumor volume.

ES cells form teratomas when grafted into a permissive environment in a recipient mouse. To determine whether Dicer affects teratoma growth or development, we injected *Dicer*$^{-/-}$ ES cells, or control heterozygous ES cells subcutaneously into nude mice. Significantly, we observed that whereas control ES cells formed teratomas almost immediately, teratomas only developed from Dicer-/- ES cells after a significant delay (Fig.1). Along with the finding that Dicer-/- ES cells fail to

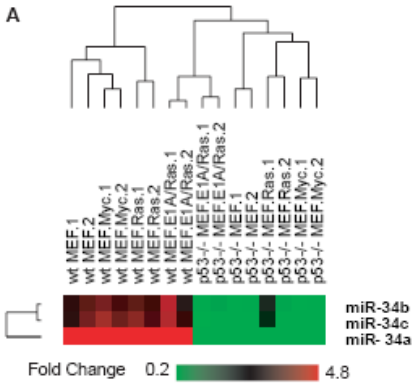

Figure 2: miR-34 expression correlates with p53 status in MEFs. miRNA expression arrays were performed in wild-type and p53-/- MEFs and a hierarchical clustering performed. The results only for miR-34 are shown here.

proliferate, these findings suggest that Dicer may protect against tumorigenesis, at least in some contexts.

The miR-34 family is controlled by p53

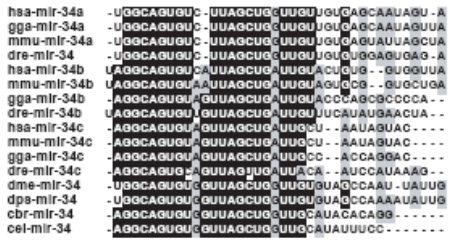

Figure 3: miR-34 is highly conserved. Multiple sequence alignment for miR-34 reveals seed conservation in vertebrates and invertebrates.

To elucidate the mechanism of Dicer's role in preventing tumorigenesis, we searched for small RNA effectors that could be involved in oncogenic pathways. The transcription factor p53 is a tumor-suppressor gene that is deleted or mutated in many human cancers. To identify miRNAs that may be part of the p53 pathway, we analyzed miRNA expression profiles in mouse embryonic fibroblasts (MEFs) in the absence and the presence of p53. Interestingly, we observed that the level of one miRNA family, that of miR-34, consistently decreased in p53-/- MEFs (Fig.2). The miR-34 family is amongst the most highly conserved miRNA gene families, with miR-34 homologs readily identifiable in the genomes of both *Drosophila* and *C.elegans* (Fig.3). In mammals, the miR-34 family is represented by three members, miR-34a – c.

miR-34 is a direct p53 target gene

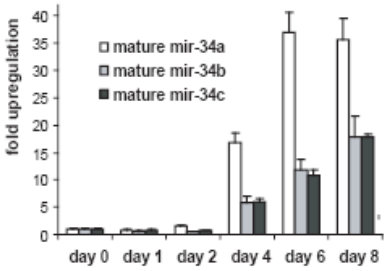

Figure 4: miR-34 levels were measured in MEFs expressing a tetracycline repressible p53 shRNA at the indicated times after doxycyclin addition.

The observation that miR-34 expression is abrogated in p53-/- cells posed the possibility that miR-34 is a direct transcriptional target of p53. To test this, we measured the levels of miR-34 in MEFs that contained a tetracycline-repressible short hairpin RNA (shRNA) against p53. Administration of doxycyclin led to an increase in miR-34 expression in a manner that was dependent upon the loss of repression of p53 (Fig.4). Furthermore, miR-34 expression was elevated in irradiated mice in a manner that was dependent upon p53 induction (Fig.5).

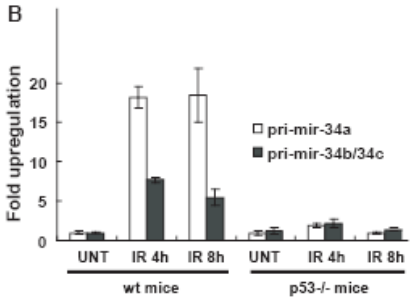

Figure 5: Levels of miR-34 primary transcripts were measured in irradiated mice that were either wild-type or p53-/-.

Next we searched the promoter regions of miR-34 primary transcripts for p53 binding sites. We were able to identify putative p53 binding sites in both miR-34 promoter regions,

and we used chromatin immunoprecipitation to confirm that p53 is enriched at these sites (Fig.6).

Key Research Accomplishments

- The development of Dicer conditional ES cell lines and mice
- Dicer is essential for the proliferation of ES cells and the absence of Dicer delays ES-cell derived teratoma onset
- The identification of a miRNA family, miR-34, in the p53 tumor suppressor pathway
- miR-34 is a direct p53 target

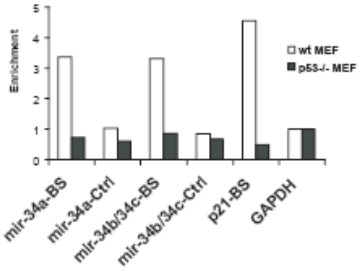

Figure 4: Chromatin immunoprecipitation of p53 at predicted p53 binding sites at miR-34 promoter regions.

Reportable outcomes

Manuscripts

Andl T, **Murchison EP**, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, Andl CD, Seykora JT, Hannon GJ and Millar SE (2006) The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr. Biol*. 2006 May 23;16(10):1041-9

Murchison EP, Partridge JF, Tam OH, Cheloufi S and Hannon GJ (2005) Characterization of Dicer deficient murine embryonic stem cells *PNAS* Aug 23;102(34):12135-40

Murchison EP and Hannon GJ (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery *Curr. Opin. Cell Biol.* Jun;16(3):223-9

Conference presentations

Murchison EP, Partridge JF, Tam OH, Cheloufi S and Hannon GJ. Characterization of Dicer deficient murine embryonic stem cells. “RNAi” Cold Spring Harbor Laboratory 2005 (poster)

Murchison EP, Hannon GJ. Proliferation defects in Dicer deficient mouse embryonic stem cells “Diverse roles of RNA in gene regulation” Beaver Run Resort, Breckenridge, Colorado 2005 (poster)

Conclusion

The important role of RNAi in cancer biology is gaining greater appreciation, and has been highlighted recently by the discoveries miRNAs with oncogenic and tumor-suppressor activities (Kent & Mendell 2006). In order to further understand the function of Dicer and small RNAs in cancer, we have produced and analyzed the tumorigenic potential of Dicer-/- ES cells, and have identified a miRNA family that is directly regulated by p53.

P53 is one of the most potent tumor suppressor genes and is found to be mutated in many types of human cancer (Lowe et al 2004; Sherr 2004). P53 is activated by a number of cellular stresses, including DNA damage and oncogenic activity (Sherr 2004). P53 is itself a transcription factor and activates a number of target genes whose activity guards the cell from growth and proliferation under inappropriate conditions. However, it has long been observed that in addition to up-regulating its target genes, p53 can also lead to the down-regulation of a number of genes in a manner that appears to be important for its downstream functions. The mechanism of this manner of regulation has been unclear.

miR-34 is a highly conserved miRNA family whose expression is dependent on p53. We have provided compelling evidence that miR-34 is a direct target of p53. This finding implicates miR-34 in the p53 pathway, and raises the possibility that down-regulation of target genes in response to p53 could be mediated by miR-34 activity.

Further studies will be required to understand the mechanism and function of Dicer's role in tumorigenesis in mammals.

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